IN THE UNITED STATES PATENT OFFICE

Seulberger et al.

U.S. Application Serial No. : 09/462,629

Filed : January 11, 2000

For : „DNA sequence encoding a hydroxyphenylpyruvate dioxygenase, and its overproduction in plants"

DECLARATION

I, Jon Falk, Dr. rer. nat., a citizen of the Federal Republic of Germany and residing at Redderkamp 15a, 24111 Kiel , Federal Republic of Germany declare as follows:

I am a biologist, having studied biology in the period of 1983 to 1990 at the Christian-Albrechts-University of Kiel, Federal Republic of Germany.

I obtained my doctor´s degree from the University in Hamburg on May 10, 1994.

I joined the University of Kiel, Botanisches Institut, Am Botanischen Garten 1-9, 24118 Kiel, Federal Republic of Germany, in January 1999.

Since January 1, 1996 I have been engaged in work in the field of plant biotechnology.

I am one of the inventors of the invention disclosed claimed in Application Serial No. 09/462,629 and am therefore familiar with the field to which the said application relates.

I intensively studied the Office Action mailed August 28, 2001 and know that the Examiner has rejected our claim 1 under 35 U.S.C. § 102(e) and § 103(a) as being anticipated and unpatentable over DellaPenna et al. (US 6,087,563).

In the new set of claims we have amended claim 1: The active claim 1 reads now:

"An isolated DNA sequence encoding a barley HPPD"

DellaPenna et al. disclose a DNA encoding an Arabidopsis HPPD, a vector, microbial host and a genetic construct containing said DNA and a transgenic plant comprising said genetic construct. It is further stated, for example in column 4, line 9 to 14, that a transgenic plant is created in which the levels of the HPPD are elevated sufficient such that production of plastoquinones, vitamin E and carotenoids are modified.

The new set of claims with amended claim 1 fulfills therefore 35 U.S.C. § 102(e). DellaPenna doesn't disclose a barley HPPD.

The new set of claims also fulfills 35 U.S.C. § 103(a): Starting from the disclosure of DellaPenna it was not possible for a person skilled in the art to come to our claimed invention without an inventive step. Arabidopsis is a dicot plant while barley is a monocot. Even if the

identity of the amino acid sequences of similar proteins in dicots and momocots are very high, the identity of the DNA sequences are often very low. Therefore it is very difficult to find a DNA sequence encoding a similar enzyme in a monocot by using a DNA probe from a dicot.

To demonstrate, that it is very unlikely to find the DNA encoding a barley HPPD by using the DNA encoding the Arabidopsis HPPD, I have investigated the identity between the amino acid sequences and the DNA Sequences. The alignment between Arabidopsis HPPD and the barely HPPD shows 57,5% identity of the amino acid sequences and only 48,1 % identity of the DNA sequences. Therefore it is very unlikely to find the DNA encoding a barley HPPD by using the DNA encoding the Arabidopsis HPPD and it was not obvious for a person skilled in the art to use a barley HPPD to increase the vitamin E level in plants.

The barley HPPD in plants has further advantageous properties: For the first time we can demonstrate a significant increase of the vitamin E level in plants and especially in crops by overexpression of a DNA encoding a barley HPPD.

To demonstrate the significant increase of the viatmin E level in plants, I have conducted the following experiments:

Test of the overexpression of the HPPD from barley in tobacco

For all transgenic line tested, the correct integration of the construct was shown by Southern blot analysis. Moreover, independent integrations could be shown for the different transgenic lines used for further analysis. All transgenic lines further tested were shown to have one intact copy of the transgene, each. Transgenic line 118 has one intact copy of the transgene and in addition a recombinant copy, which is probably not functional.

Over expression of the HPPD was tested by germination of seeds of transformed plants in microtiter plates in the presence of Mikado, a HPPD specific herbicide. Seeds were first surface sterilized with ethanol and then incubated aseptically on blotting paper soaked in MS medium (Murashige and Skoog 1962) with 0, 0.3, 1.5 or 3 µM sulcotrione, a herbicide specifically inhibiting the HPPD (Secor 1994), in a growth chamber at 24 °C and a daily light/dark regime of 16/8 hrs. After seven days the phenotype could be seen and resistant green seedlings were further cultivated an soil for further experiments.

Among several transgenic tobacco lines an up to tenfold higher resistance towards Mikado was found. Four different transgenic lines (No. 39, 70, 95, and 118) with different levels of HPPD over expression were chosen for further analysis of the vitamin E content in leaves and seeds.

Four different individual plants for each transgenic line were further grown in a climate chamber for ten weeks until they reached a height of about 120 cm just before the beginning of blooming. The plants were grown at a temperature of 26°C and 16 hours of light (500

µmol x sec^{-1} x m^{-2}) a day. The youngest fully expanded leaf were chosen for further analysis of the vitamin E content.

100 to 200 mg of powdered frozen plant material were extracted with 2x volume n-heptane-2-propanol (99.5+0.5) at -20°C for at least 24 hours. After centrifugation twice 20 µl of the clear supernatant were analysed by HPLC using a LiChrosphere Si 100 (5µm) column (10 x 250 mm) with n-heptane-2-propanol (99.5+0.5) as elutant at a flow rate of 1.5 mL/min. Vitamin E forms were detected and quantified using a fluorescence detector (model RF10AXL, Shimadzu) set at $\lambda_{excitation}$=290nm and $\lambda_{emission}$=328nm. Tocopherol and tocotrienol standards were purchased from Merck (Darmstadt, Germany) to calibrate the system and verify the identity of individual peaks. Additionally the identity of vitamin E in the probes was verified by measuring the fluorescence emission at a second wavelength ($\lambda_{emission}$=370nm). As for the vitamin E standards a defined decrease in peak area is found specific for vitamin E (Ivanov and Aitzetmueller 1995).

In tobacco leaves only α- and at a much lower level γ-tocopherol are present (data not shown). Despite the higher expression rate of the HPPD in the transgenic lines no significant increase in vitamin E content in leafs were observed. Therefore, the homogentisate produced additionally is not used to synthesize more vitamin E.

At the beginning of flowering the plants were transferred to a greenhouse for seed set. After ripening of the seeds probes were taken from three individual plants for each transgenic line analysed.

Different vitamin E forms are present in tobacco leaves and seeds. In tobacco seeds usually mainly γ-tocopherol and γ-tocotrienol are found, although α-tocopherol and α-tocotrienol are present at very low amounts (s. Fig. 1). In seeds of HPPD over expressing plants an up to twofold increased accumulation of vitamin E can be found. The transgenic line with the greatest increase in vitamin E content in their seeds were also the most resistant against the herbicide Mikado (tenfold). Therefore, in seeds additionally produced homogentisate is at least partially used to accumulate more vitamin E. Interestingly, the tocopherol/tocotrienol ratio is not changed in the HPPD over expressing plants. Therefore, the homogentisate is equally used to produce both vitamin E forms .

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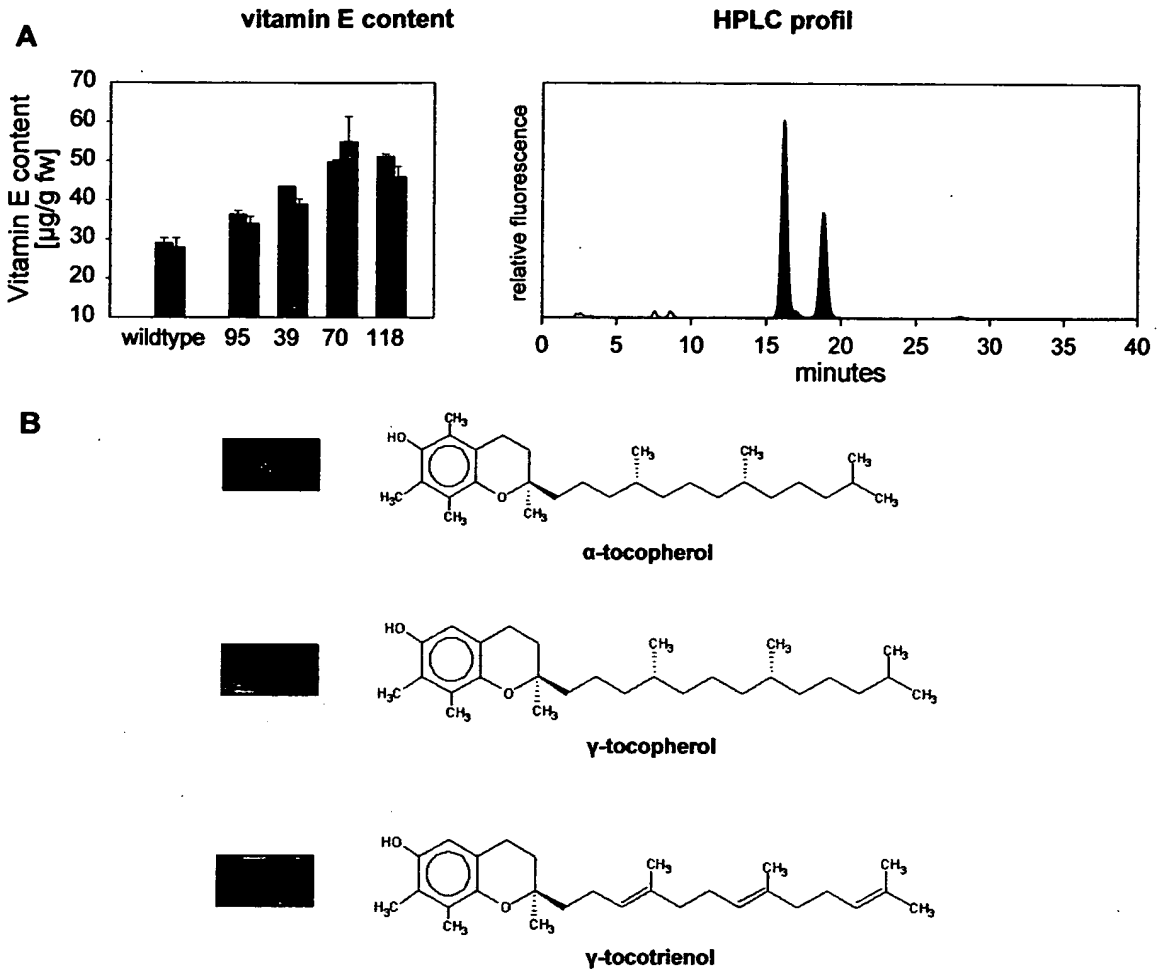

Fig. 1: Analysis of the vitamin E content in seeds (A) of wildtype and different HPPD over expressing tobacco plants. (B) Molecular structure of vitamin E forms found in leaves and seeds of tobacco.

I further declare that all statements made herein of my own knowledge are true and that statements made on information or belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment , or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed at 24118 Kiel, Germany, January 22, 2002

Signature of Declarant